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Safety of heat-killed *Mycobacterium setense manresensis* as a novel food pursuant to Regulation (EU) 2015/2283

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Abstract

Following a request from the European Commission, the EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) was asked to deliver an opinion on heat-killed *Mycobacterium setense manresensis* as a novel food (NF) pursuant to Regulation (EU) 2015/2283. The NF is an encapsulated ingredient composed of 200 mg mannitol and $\leq 10^5$ heat-killed, freeze-dried *M. setense manresensis*. The information provided on the efficacy of the heat inactivation process demonstrates that the applied thermal process effectively kills all *M. setense manresensis*. The Panel considers that the NF is sufficiently described and characterised. The NF is intended by the applicant to be marketed exclusively in food supplements (gelatine capsules) for the general adult population excluding, children, pregnant and lactating women. The NF is not intended to be an alternative to standard treatment against tuberculosis. The applicant proposed an intake of one capsule (with $\leq 10^5$ heat-killed, freeze-dried *M. setense manresensis*) for 14 consecutive days and a minimum of 6 months with no consumption of the NF, before another intake for fourteen days may follow. *M. setense* is not considered to be a suitable microorganism species for the qualified presumption of safety (QPS). Genetic analyses of the genome indicate the absence of the ability to produce exotoxins. The Panel considers that consumption of heat-killed *M. setense manresensis* would not contribute to the pool of transmissible antimicrobial resistance genes already present in the intestinal microbiota. The Panel concludes that the NF is safe under the proposed conditions of use.

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Keywords: *Mycobacterium setense manresensis*, supplement, novel food, ingredient, safety

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Summary

Following a request from the European Commission, the EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) was asked to deliver a scientific opinion on a supplement containing heat-killed *Mycobacterium setense manrensensis* as a novel food (NF) pursuant to Regulation (EU) 2015/2283. The assessment of the safety of this NF, which follows the methodology set out in the EFSA Guidance on the preparation and presentation of an application for authorisation of a novel food[1] Regulation (EU) 2015/2283 and in the Commission Implementing Regulation (EU) 2017/2469, is based on the data supplied in the application, and information submitted by the applicant following the European Food Safety Authority (EFSA) requests for supplementary information and additional data identified by the Panel.

The NF is an encapsulated ingredient composed of 200 mg mannitol and $\leq 10^5$ heat-killed, freeze-dried *M. setense manresensis.* The production process is sufficiently described in the dossier and does not raise safety concerns. The information provided on the efficacy of the heat inactivation process, i.e. a culture method which allows the growth of stressed bacilli which theoretically may have survived the heat treatment, demonstrates that the applied thermal process effectively kills all *M. setense manresensis*. The Panel considers that the NF is sufficiently described and characterised.

The NF is intended by the applicant to be marketed exclusively in food supplements (gelatine capsules) for the general adult population. The applicant proposes to exclude pregnant and lactating women, and children from the target population for precautionary reasons. The applicant also indicates that the NF is not intended as an alternative to standard treatment against tuberculosis. The applicant proposed an intake of one capsule per day (with $\leq 10^5$ heat-killed, freeze-dried *M. setense manresensis*) for 14 consecutive days and indicated that this limitation is for precautionary reasons, considering the exposure duration (i.e. 14 days) of the presented human trial. In response to an EFSA request, the applicant also proposed a minimum of 6 months with no consumption of the NF, before another intake for 14 days may follow. The applicant indicated that there was no underlying scientific rationale or safety concerns for this duration of a consumption free interval, but this proposal was again for precautionary reasons.

According to the EFSA QPS Statement (2018), species of this *Mycobacterium fortuitum* group to which *M. setense manresensis* belongs, have been reported to cause skin, bone and joint infections, and mycolic acids of mycobacteria are recognised to induce granulomatous lesions. Therefore *M. setense* was found not to be a suitable microorganism species for the qualified presumption of safety (QPS). Genetic analyses of the genome indicate the absence of the ability to produce exotoxins. A study report was presented on an animal experiment with severe combined immunodeficiency (SCID) mice, in which viable *M. setense manresensis* showed no virulence in this test.

The Panel considers that the resistance of *M. setense manresensis* against various antimicrobials is intrinsic and non-transmissible. The Panel concludes therefore that consumption of heat-killed *M. setense manresensis* would not contribute to the pool of transmissible antimicrobial resistance genes already present in the intestinal microbiota.

In addition, a randomised controlled trial (RCT) was performed with the NF (Montané et al., 2017). No statistically significant differences were found in the occurrence of adverse events between the placebo and the two treatment groups. Because of the limitations of this study (e.g. the low dose, the low number of subjects, number of endpoints, the age range of the subjects and the shortness of the study), the Panel considers the study of limited value for the safety assessment of the NF.

The Panel concludes that the NF is safe under the proposed conditions of use.

[1] EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), Turck D, Bresson J-L, Burlingame B, Dean T, Fairweather-Tait S, Heinonen M, Hirsch-Ernst KI, Mangelsdorf I, McArdle H, Naska A, Neuhäuser-Berthold M, Nowicka G, Pentieva K, Sanz Y, Siani A, Sjödin A, Stern M, Tomé D, Vinceti M, Willatts P, Engel K-H, Marchelli R, Pöting A, Poulsen M, Salminen S, Schlatter J, Arcella D, Gelbmann W, de Sesmaisons-Lecarré A, Verhagen H and van Loveren H, 2016. Guidance on the preparation and presentation of an application for authorisation of a novel food in the context of Regulation (EU) 2015/2283. EFSA Journal 2016;14(11):4594, 24 pp. https://doi.org/10.2903/j.efsa.2016.4594

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1. Introduction

1.1. Background and Terms of Reference as provided by the European Commission

On 23 February 2018, the company Nutraveris/Laboratorio Reig Jofre, S.A. submitted a request in accordance with Article 10 of Regulation (EU) 2015/2283[2] to place on the market a supplement containing heat-killed bacteria *Mycobacterium (M.) setense manresensis* as a novel food (NF).

In accordance with Article 10 (3) of Regulation (EU) 2015/2283, EFSA shall give its opinion as to whether the update of the Union List referred to in Article 10 (1) is liable to have an effect on human health.

2. Data and methodologies

2.1. Data

The safety assessment of this NF is based on data supplied in the application and information submitted by the applicant following the European Food Safety Authority (EFSA) requests for supplementary information.

Administrative and scientific requirements for NF applications referred to in Article 10 of Regulation (EU) 2015/2283 are listed in the Commission Implementing Regulation (EU) 2017/2469.[3]

A common and structured format on the presentation of NF applications is described in the EFSA guidance on the preparation and presentation of a NF application.[4] As indicated in this guidance, it is the duty of the applicant to provide all of the available (proprietary, confidential and published) scientific data, including both data in favour and not in favour for supporting the safety of the proposed NF.

This NF application includes a request for protection of proprietary data in accordance with Article 26 of Regulation (EU) 2015/2283. Data claimed to be proprietary by the applicant include: all the studies and scientific evidence provided to characterise the product, to determine the potential antibiotic resistance, the risk of horizontal gene transfer, the genotoxicity, the oral toxicity and the virulence of *M. setense manresensis*.

2.2. Methodologies

The assessment follows the methodology set out in the EFSA guidance on NF applications and the principles described in the relevant existing guidance documents from the EFSA Scientific Committee. The legal provisions for the assessment are laid down in Article 11 of Regulation (EU) 2015/2283 and in Article 7 of the Commission Implementing Regulation (EU) 2017/2469.

This assessment concerns only risk that might be associated with consumption of the NF under the proposed conditions of use, and is not an assessment of the efficacy of NF with regard to any claimed benefit.

3. Assessment

3.1. Introduction

The NF is a capsule that consists of heat-killed, freeze-dried *M. setense manresensis* bacteria and mannitol as bulking agent. The applicant proposed that the NF will be used as a food supplement.

[2] Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001 (2013/0435 (COD). OJ L 327, 11.12.2015, p. 1–22.

[3] Commission Implementing Regulation (EU) 2017/2469 of 20 December 2017 laying down administrative and scientific requirements for applications referred to in Article 10 of Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods. OJ L 351, 30.12.2017, pp. 64–71.

[4] EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), Turck D, Bresson J-L, Burlingame B, Dean T, Fairweather-Tait S, Heinonen M, Hirsch-Ernst KI, Mangelsdorf I, McArdle H, Naska A, Neuhauser-Berthold M, Nowicka G, Pentieva K, Sanz Y, Siani A, Sjoedin A, Stern M, Tomé D, Vinceti M, Willatts P, Engel K-H, Marchelli R, Pöting A, Poulsen M, Salminen S, Schlatter J, Arcella D, Gelbmann W, de Sesmaisons-Lecarre A, Verhagen H and van Loveren H, 2016. Guidance on the preparation and presentation of an application for authorisation of a novel food in the context of Regulation (EU) 2015/2283. EFSA Journal 2016;14(11):4594, 24 pp. https://doi.org/10.2903/j.efsa.2016.4594

3.2. Identity of the NF

The NF is an encapsulated ingredient composed of 200 mg mannitol and $\leq 10^5$ heat-killed, freeze-dried *M. setense manresensis* (Family: Mycobacteriaceae; Genus: *Mycobacterium*; Species: *Setense*; Strain: *Manresensis*).

This strain of *M. setense* was isolated from the Cardener River in Manresa, Catalonia, Spain and is identified by the analysis of its 16S rRNA sequence and phenotypically characterised (Rech et al., 2015). The whole genome sequence of *M. setense manresensis* has been deposited in GenBank under the accession number JTLZ00000000. According to the application, the subject of this application, *M. setense manresensis*, is deposited in the Colección Española de Cultivos Tipo (CECT), under number CECT 8638 and in the Belgian coordinated collections of microorganisms/*Mycobacterium* catalogue (BCCM/ITM), under number WSLA13#18.

3.3. Production process

The applicant provided a detailed description of the production process of the NF which included information on the controls and checkpoints applied at the production steps. The production of *M. setense manresensis* follows a HACCP certification. The freeze-drying and solid-phase mixing are performed in a facility certified ISO 9001:2008.

The manufacturing process of the NF starts with the cultivation of *M. setense manresensis*. The bacterium grows in various fermenters, increasing in size during the process. Growth inactivation and killing of the bacteria occur by heating the final bacterium culture at 80°C for 32 min. Then, a centrifugation step is applied to obtain the concentrated heat-killed *M. setense manresensis*. A freeze-drying step is then performed to obtain the dried heat-killed bacteria which are subsequently diluted and mixed in with food-grade mannitol by a factor of 1/400,000 to obtain a bulk formulation with a concentration of 0.00025% heat-killed *M. setense manresensis* bacteria.

Information on the production process indicates that this 0.00025% bulk formulation is then subject to an in-house analysis method based on a quantitative enzyme-linked immunosorbent assay (ELISA) test. Information including the protocol and the validation of this ELISA has been provided to EFSA. Depending on the results (i.e. the number of heat-killed bacteria), a final dilution step and mixing with food-grade mannitol is applied at a dilution factor which is required to obtain $\leq 5 \times 10^5$ heat-killed ($\leq$ 625 ng) *M. setense manresensis* per gram. Two hundred milligram of this formulation is encapsulated in gelatine, resulting in $\leq 10^5$ heat-killed bacteria ($\leq$ 125 ng) per capsule. The dilution factor for this last dilution step with mannitol could range from 4 to 100 according to the applicant.

The Panel considers that the data provided sufficient information with respect to the production process of the NF.

3.4. Compositional data

The applicant provided batch-to batch analysis of the 0.00025% bulk formulation from five batches presented in Table 1.

Table 1: Batch-to-batch analysis of the 0.00025% formulation

Parameter	Specification	Batch				
		C14	**A15**	**A14**	**A16**	**B15**
Appearance	White homogeneous powder	Complies	Complies	Complies	Complies	Complies
Water content (%)	$\leq$ 1.7%	0.20	0.40	0.27	0.60	0.30
pH	4–7	4.83	4.49	6.1	4.97	4.65
Quantification (with in-house ELISA method)	2×10^6–5×10^7 heat-killed *Mycobacterium setense manresensis*/g	7.01×10^6	2.84×10^7	2.86×10^7	2.26×10^7	7.15×10^6
Microbiological data						
TAMC	$\leq 10^3$ CFU/g	44	6	13	8	0
TYMC	$\leq 10^3$ CFU/g	0	0	0	0	0
Escherichia coli	Absence/g	0	0	0	0	0

Heavy metals		C14	A15	2C14	A16	B15
Mercury (Hg)	< 0.005 mg/kg	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
Arsenic (As)	< 0.01 mg/kg	< 0.1	< 0.1	< 0.1	< 0.1(1)	< 0.1
Lead (Pb)	< 0.05 mg/kg	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Cadmium (Cd)	< 0.01 mg/kg	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

CFU: colony forming units, ELISA: enzyme-linked immunosorbent assay; TAMC: total aerobic microbial count, TYMC: total yeast and mould counts.
(1): Method of analysis for batch A16: method analogue §64 LFGB L 00.0-19/3.

The range for the number of heat-killed *M. setense manresensis* bacteria in this 0.00025% bulk formulation among the five batches was 7.01×10^6–2.86×10^7 per gram. According to the information provided in the production process and in order to achieve a final concentration of 5×10^5 per gram of heat-killed *M. setense manresensis*, the required dilution factor to be applied for these 5 batches would range from 12.7 to 57.2.

The Panel considers that the information provided on the composition of the NF is sufficient and does not raise safety concerns.

3.4.1. Stability

The stability of the NF stored sealed in bags and in capsules was tested for 24 months at 30°C with 75% humidity. There were no significant changes over this time among the tested parameters which included the pH-value, water content, microbiological counts (total aerobic count, total yeasts and moulds count and *Escherichia coli*) and appearance.

The Panel considers that the data provided sufficient information with respect to the stability of the NF.

3.5. Specifications

The applicant provided specifications for the NF presented in Table 2.

Table 2: Specifications of the NF (per gram)

Parameter	Acceptance criteria	Method
Appearance	White homogeneous powder	Visual inspection
Water content (%)	≤ 1.7%	Eur. Ph. 2.5.32
pH	4–7	Eur. Ph. 2.2.3
Heat-killed *Mycobacterium setense manresensis*	≤ 625 ng ≤ 5×10^5 heat-killed *M. setense manresensis*	In-house method (ELISA) using polyclonal antibodies applied on the results of the 0.00025% formulation. Depending on the results, the 0.00025% formulation is subsequently diluted with mannitol with a factor between 4 and 100 in order to achieve ≤ 5×10^5 heat-killed *M. setense manresensis*
Microbiological control		
TAMC	≤ 10^3 CFU/g	Eur. Ph. 5.1.4
TYMC	≤ 10^2 CFU/g	Eur. Ph. 5.1.4
Escherichia coli	Absence/g	Eur. Ph. 5.1.4
Heavy metal testing		
Mercury (Hg)	≤ 0.005 mg/kg	EN 15763:2009
Arsenic (As)	≤ 0.1 mg/kg	EN 15763:2009
Lead (Pb)	≤ 0.05 mg/kg	EN 15763:2009
Cadmium (Cd)	≤ 0.01 mg/kg	EN 15763:2009

CFU: colony forming units, ELISA: enzyme-linked immunosorbent assay; TAMC: total aerobic microbial count, TYMC: total yeast and mould counts.

The Panel notes that the applicant provided batch-to-batch analysis for five batches of the 0.00025% bulk formulation of the NF, not on the final formulation (i.e. after the final dilution step). Depending on the results (number of heat-killed *M. setense manresensis* analysed by the quantitative ELISA), the 0.00025% formulation requires a final dilution and mixing with mannitol at a dilution

factor between 4 and 100 to be applied to achieve the specification parameter of $\leq 5 \times 10^5$ heat-killed *M. setense manresensis*.

Following a request from EFSA, the applicant provided additional information on experimental data to demonstrate consistent production process and compliance with the specification regarding the absence of viable mycobacteria. In order to show the growth of stressed bacilli which may theoretically survive the heat treatment, plates with Middlebrook 7H11 agar were incubated for eight weeks. Nine samples for four independent batches of the NF have been used. For each sample, 10 g of product were diluted in 100 mL of sterile water. A total of 10 plates for each sample were spread with 1 mL of this solution (i.e. for a total of 90 plates). The presence of CFU was then assessed weekly for eight weeks. After eight weeks of incubation at 35°C, no CFU of *M. setense manresensis* could be detected in any of the samples assessed.

The pesticide residues in the NF were also analysed using a gas chromatography with mass spectrometry (GC–MS)-based protocol (SFLKA–5: internal method) and a liquid chromatography with tandem mass spectrometry (LC–MS/MS)-based protocol (reference method: LFGB L 00.00-113) for complex food matrices and the levels were below the limits of quantification.

The Panel considers that the information provided on the specifications and the batch-to-batch variability of the NF is sufficient and does not raise safety concerns.

3.6. History of use of the NF and/or of its source

The NF has no history of consumption in the European Union (EU).

As indicated in the publication of a human trial with this NF (Montané et al., 2017), the occurrence of non-tuberculous mycobacteria including mycobacteria of the *M. fortuitum* complex (a group of related *Mycobacterium* to which also the species *M. setense* belongs) from tap water has been reported for different continents including also European countries (Martín Casabona and Rosselló Urgell, 2000; Falkinham, 2009; Fernandez-Rendon et al., 2012; Imwidthaya et al., 1989; Kubalek and Mysak, 1996; Nasr-Esfahani et al., 2012; Moghim et al., 2012; Primm et al., 2004; Scarlata et al., 1985).

3.7. Proposed uses and use levels and anticipated intake

3.7.1. Target population

The target population for the NF as proposed by the applicant is the general adult population. The applicant indicated that the NF is not intended as an alternative to standard treatment against tuberculosis. The applicant proposes to exclude pregnant and lactating women, and children from the target population. In response to an EFSA request regarding the rationale for this proposed restriction, the applicant stated that his proposal was for precautionary reasons, stating that there are no data supporting the safety of the NF ingredient in these population groups.

3.7.2. Proposed uses and use levels

The NF will be used in food supplements only. The applicant proposed a daily intake of one 200 mg capsule of the NF containing $\leq 10^5$ ($\leq$ 125 ng) of heat-killed *M. setense manresensis* in mannitol for 14 days. The applicant indicated that this limitation of 14 days is not based on a safety concern, but is proposed for precautionary reasons, considering that this duration corresponds to the duration of intake of the NF in the presented human trial (Montané et al., 2017). In response to an EFSA request, the applicant proposes that between two 14-day periods of intakes, there should be a minimum of 6 months with no consumption of the NF. For this restriction, the applicant indicated no scientific rationale and also no safety concerns, but that also this proposal was for precautionary reasons.

3.8. Absorption, distribution, metabolism and excretion (ADME)

No relevant data have been provided on ADME.

3.9. Nutritional information

Based on the nature and the intended use levels of the NF, the Panel considers that the NF has no nutritional relevance in human.

The Panel considers that consumption of the NF is not nutritionally disadvantageous.

3.10. Toxicological information

The Panel considers that there is no rationale for applying the default toxicological testing approach established for chemicals such as genotoxicity testing, subacute and subchronic testing to a novel food consisting of heat-killed mannitol-diluted bacteria grown in medium which does not give rise to toxicological concerns.

3.10.1. Microbiological information

According to Rech et al. (2015), the microorganism subject of this application represents a new strain of the species *M. setense.* The EFSA QPS statement (2018) notes that *M. setense* is a member of the *M. fortuitum* complex, which is well known by its ability to cause skin, bone and joint infections (Yu et al., 2013) and mycolic acids of mycobacteria are recognised to induce granulomatous lesions (Fujita et al., 2007). Therefore *M. setense* cannot be considered a suitable microorganism species for the qualified presumption of safety (QPS) status because there are significant safety concerns (EFSA BIOHAZ Panel, 2019). According to German TRBA (technical rules for biological agents), this species falls into risk group 2 and 'can cause human disease, might be a hazard to workers, unlikely to spread to the community' (Directive 2000/54/EC[5]).

Rech et al. (2015) have published the draft genome sequence on *M. setense manresensis,* and compared it with the genome of *M. setense* type strain DSM 45070. DSM 45070 was first isolated from a patient with soft tissue infection and osteitis (Lamy et al., 2008). The strain *manresensis* shares a 98.5% of sequence identity with DSM 45070, and has 3.7% of its genome heterologous to DSM 45070 while 6.7% of the DSM 45070 genome is without homology in *manresensis* (Rech et al., 2015). *Manresensis* differs from DSM 45070 by a 0.2-MB smaller genome size and fewer genes.

To investigate potential virulence of *M. setense manresensis*, a bioinformatics study has examined the putative genes associated with virulence in the whole genome of *M. setense manresensis* (Comas, 2016a; unpublished). The applicant has used the Virulence Factor Database (VFDB[6]) to identify potential virulence factors encoded in the genome of *M. setense manresensis*. In addition, EFSA performed a search on the virulence factors of the strain using the Patric database.[7] Both searches identified several virulence factors associated with metabolism of the strain and environmental stress, which are considered not relevant for heat-killed bacteria. In these searches genes encoding for exotoxins were not identified.

Virulence assay in SCID mice

A study to evaluate the potential virulence of viable *M. setense manresensis* (Vilapana, 2014; unpublished) was done in severe combined immunodeficiency (SCID) mice. The animals were divided into six groups (6 animals per group), two of which received intravenously 10^5 or 10^6 CFU of viable *M. bovis* BCG as a positive control, with the other four receiving by the same route 10^5, 10^6 or 10^7 CFU of *M. setense manresensis*. All animals in the positive control groups died within 80–130 days. No mortality, no weight loss or signs of suffering were noted in the *M. setense manresensis*-treated mice. No virulence has been identified for the viable *M. setense manresensis* in this study.

Susceptibility to antimicrobials

The applicant also provided examinations on the antimicrobial resistance (AMR) (Comas, 2016b; unpublished) and susceptibility of *M. setense manresensis* (Esteban, 2015; unpublished). Two databases, i.e. the Antibiotic Resistance Gene-ANNOTation and the Antibiotic Resistance Gene Database (ARDB), have revealed that the bacterium was resistant to aminoglycosides, bacitracin, phenicols and tetracyclines (among others). Furthermore, a susceptibility testing was conducted in two systems, i.e. a commercial broth microdilution including 15 different antibiotics (minimum inhibitory concentrations (MICs)) and E-strip susceptibility testing, using five antibiotics. The results showed that *M. setense manresensis* was sensitive to amikacin, quinolones, cefoxitin, imipenem and tigecycline, but resistant to other β-lactam antibiotics, tetracyclines (doxycycline and minocycline) and to clarithromycin.

[5] Directive 2000/54/EC of the European Parliament and of the Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work (seventh individual directive within the meaning of Article 16(1) of Directive 89/391/EEC).
[6] http://www.mgc.ac.cn/VFs/main.htm
[7] https://www.patricbrc.org/

Upon EFSA request about the intrinsic or acquired character of the AMRs present in *M. manresensis*, the applicant provided scientific literature supporting the intrinsic character of most of the antibiotics resistance genes that are shared with other members of the *M. fortuitum* complex. However, no evidence was provided to confirm that the resistance of the *M. setense manresensis* to phenicols is intrinsic (and not acquired). Therefore, the applicant was requested to elaborate on this specific phenicols resistance in accordance to Section 2.2 of the EFSA FEEDAP Panel (2018).

The applicant addressed the EFSA request by analysing the MIC of *M. setense manresensis* towards different antibiotics including phenicols, in accordance with CLSI recommendations. In addition, following a first analysis of putative genes associated to drug resistance in *M. setense manresensis*, a new search was performed in order to analyse the gene sequence for chloramphenicol-associated genes identified in the first study report. Three candidate genes were identified, all associated to the same coding sequence (JTJW01000010.1_10_117). The three genes are referred to the same predicted protein, which can be identified as a 'Major Facilitator Superfamily' (MFS), a membrane transport protein that is ubiquitous across life domains, and involved in the transport of various substances. Additional analysis showed that this gene is at its expected taxonomic position for *M. setense manresensis*, in comparison to *M. setense*. Therefore, it is concluded that the gene is very likely to be intrinsic.

To provide additional evidence of the intrinsic character (not transmissible) of the AMR of *M. setense manresensis*, the applicant referred to the lateral gene transfer (LGT) occurrence associated with defence mechanisms present in *Mycobacterium* genomes that are of unknown functions or associated with energy production and conversion (Fedrizzi et al. 2017). The contribution of LGT to the genetic material of *M. fortuitum* ranks from 0.5% to 0.75%. Hence, the risk of LGT in *M. manresensis* can be considered low. Moreover, Nessar et al. (2012) indicated that the AMR genes are acquired by spontaneous mutation and they are non-transmissible. No plasmids have been identified in *M. manresensis*.

The Panel considers that the resistance of *M. setense manresensis* against various antimicrobials is intrinsic and non-transmissible. The Panel concludes therefore that consumption of heat-killed *M. setense manresensis* would not contribute to the pool of transmissible AMR genes already present in the gut bacterial population.

3.10.2. Human data

A double-blinded, randomised, placebo-controlled trial (RCT) was performed to study the tolerability and immunological endpoints related to tuberculosis, i.e. *M. tuberculosis*- specific regulatory T-helper cells (Tregs, i.e. $CD25^+CD39^-$, $CD25^+CD39^+$, $CD25^-CD39^-$, $CD25^-CD39^+$) (Montané et al., 2017). According to the article, the study was conducted in compliance with the Helsinki declaration and the Good Clinical Practice guidelines of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. In total 51 healthy adults between 22 and 42 years of age were enrolled, of whom 30 were latent tuberculosis infection negative ($LTBI^-$) and 21 were LTBI-positive ($LTBI^+$). The randomisation was stratified by this characteristic. Distilled water was used as a placebo (n = 18, 10 $LTBI^+$ and 8 $LTBI^-$), and 10^4 and 10^5 heat-killed *M. setense manresensis* for the low (n = 16, 10 $LTBI^+$ and 6 $LTBI^-$) and high dose (n = 17, 10 $LTBI^+$ and 7 $LTBI^-$), were administered, respectively. The participants received one of the test substances once per day for 14 days and were followed for 6 weeks beginning with the administration of the first dose. Physical examinations and blood tests (erythrocytes, leucocytes and platelets, haemoglobin, haematocrit, fasting glucose, aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transpeptidase, alkaline phosphatase, total bilirubin, direct and indirect bilirubin, urea N, creatinine, glomerular filtration rate, sodium and potassium) were performed. Both gastrointestinal and non-gastrointestinal adverse events were recorded on the basis of spontaneous reporting of the study subjects and regular questions of the study investigators. No statistically significant differences were found in the physical examinations, results of the blood tests and the occurrence of adverse events between the placebo and the two treatment groups.

The authors of this article reported for both ($LTBI^+$ and $LTBI^-$) groups of volunteers who had received either 10^4 or 10^5 heat-killed *M. setense manresensis* an increase of $CD25^+CD39^-$ and $CD25^+CD39^+$ Treg cells reactive to protein purified derivative (PPD; a reagent used for the tuberculosis skin test). However, these reported increases were not consistent across the treatment groups and time and were observed only for a few within group analyses and not as compared to placebo. The Panel considers that the immune-related results do not raise safety concerns.

Because of the limitations of the study (e.g. the low dose, the low number of subjects, number of endpoints, the age range of the subjects, and the shortness of the study), the Panel considers the study of limited value for the safety assessment of the NF.

3.11. Allergenicity

There is no evidence from the literature on allergenicity of mycobacteria. The confidential description of the production process indicates the use of potentially allergenic proteins (bovine albumin in the growth medium), which are expected to be removed during the wash step by centrifugation.

The Panel considers that the allergenicity of the NF is low.

4. Discussion

The Panel considers that the NF is sufficiently described and characterised. On the basis of experimental data provided to validate the inactivation step applied in the production process, the Panel considers that the NF does not contain live bacteria. The production process is sufficiently described in the dossier and does not raise safety concerns. The information provided on the efficacy of the heat inactivation process, i.e. a culture method which allows the growth of stressed bacilli which theoretically may have survived the heat treatment, demonstrates that the applied thermal process effectively kills all *M. setense manresensis*.

The NF is intended by the applicant to be marketed exclusively in food supplements (gelatine capsules) for the general adult population. The applicant proposed to exclude pregnant and lactating women, and children from the target population for precautionary reasons. The applicant also indicated that the NF is not intended as an alternative to standard treatment against tuberculosis. The applicant proposed an intake of one capsule daily (with $\leq 10^5$ heat-killed, freeze-dried *M. setense manresensis*) for 14 consecutive days and indicated that this limitation is for precautionary reasons, considering the exposure duration (i.e. 14 days) of the presented human trial. In response to an EFSA request, the applicant also proposed a minimum of 6 months with no consumption of the NF, before another intake for 14 days may follow. The applicant indicated that there was no underlying scientific rationale or safety concerns for this duration of a consumption free interval, but this proposal was again for precautionary reasons.

According to the EFSA QPS Statement (2018), species of this *M. fortuitum*-group to which *M. setense manresensis* belongs, have been reported to cause skin, bone and joint infections, and mycolic acids of mycobacteria are recognised to induce granulomatous lesions. Therefore *M. setense* was found not to be a suitable microorganism species for the QPS. Genetic analyses of the genome indicate the absence of the ability to produce exotoxins. A study report was presented on an animal experiment with SCID mice, in which viable *M. setense manresensis* showed no virulence in this test.

The Panel considers that the resistance of *M. setense manresensis* against various antimicrobials is intrinsic and non-transmissible. The Panel concludes therefore that consumption of heat-killed *M. setense manresensis* would not contribute to the pool of transmissible AMR genes already present in the intestinal microbiota.

In addition, in a RCT performed with the NF (Montané et al., 2017), no statistically significant differences were found in the occurrence of adverse events between the placebo and the two treatment groups. Because of the limitations of the study (e.g. the low dose, the low number of subjects, number of endpoints, the age range of the subjects and the shortness of the study), the Panel considers the study of limited value for the safety assessment of the NF.

5. Conclusions

The Panel concludes that the NF is safe under the proposed conditions of use (which includes the restrictions that the NF should not be consumed longer than for 14 consecutive days and that between two 14-day periods of intakes, there should be a minimum of 6 months with no consumption of the NF).

The Panel could not have reached the conclusion on the safety of the NF under the proposed conditions of use without the following data claimed as proprietary by the applicant: the studies and scientific evidence provided to characterise the product (Rech et al., 2015; batch testing), the validation of the heat inactivation, to determine the potential antibiotic resistance and the risk of horizontal gene transfer (Comas, 2016b, unpublished; Esteban, 2015, unpublished), the virulence of *M. setense manresensis* (Vilapana, 2014; unpublished).

Steps taken by EFSA

1) Letter from the European Commission to the European Food Safety Authority with the request for a scientific opinion on the safety of heat-killed *Mycobacterium setense manresensis*. Ref. Ares(2018)3353838, dated 25. June 2018.
2) On 25 June 2018, EFSA received a valid application from the European Commission on heat-killed *Mycobacterium setense manresensis* as NF, which was submitted by Laboratorio Reig Jofre, S.A., and the scientific evaluation procedure started.
3) On 11 December 2018, 2 May and 6 September 2019, EFSA requested the applicant to provide additional information to accompany the application and the scientific evaluation was suspended.
4) On 28 February, 19 August and 9 September 2019 additional information was provided by the applicant and the scientific evaluation was restarted.
5) During its meeting on 18-19 September 2019, the NDA Panel, having evaluated the data, adopted a scientific opinion on the safety of heat-killed *Mycobacterium setense manresensis* as a NF pursuant to Regulation (EU) 2015/2283.

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Abbreviations

ADME	absorption, distribution, metabolism and excretion
AMR	antimicrobial resistance
ARDB	Antibiotic Resistance Gene Database
CECT	Colección Española de Cultivos Tipo
CLSI	The Clinical & Laboratory Standards Institute
ELISA	enzyme-linked immunosorbent assay
GC–MS	gas chromatography with mass spectrometry
HACCP	Hazard Analysis and Critical Control Points
LC–MS/MS	liquid chromatography with tandem mass spectrometry
LGT	lateral Gene Transfer
LTBI	latent tuberculosis infection
MFS	major facilitator superfamily
MIC	minimum inhibitory concentration
NDA	EFSA Panel on Nutrition, Novel Foods and Food Allergens
NF	novel food
QPS	qualified presumed safety
PPD	protein purified derivative
RCT	randomised controlled trial
SCID	severe combined immunodeficiency
TRBA	technique rules for biological agents
VFDB	Virulence Factor Database